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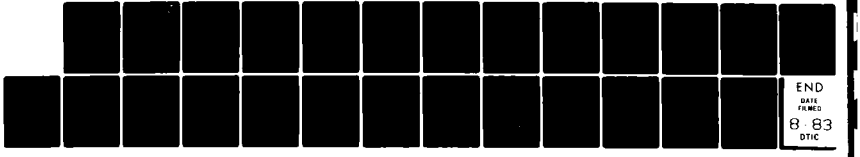

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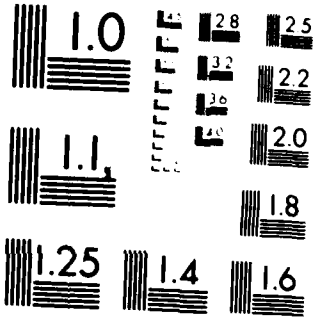

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Annual Report

Saint Louis Encephalitis Temperature-Sensitive Mutants

Thomas A. Brawner, Ph.D.

September 1980

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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ABSTRACT

Conditional lethal temperature-sensitive (ts) mutants are defined by their inability to replicate at a nonpermissive temperature. Generally, this temperature is above 37°C. Temperature-sensitive mutants have often been used to examine the molecular mechanisms of viral replication and more recently they have been used as vaccine strains due to their reduced virulence. As a first step in the development of a population of temperature-sensitive mutants, a heat resistant viral clone was developed by multiple cycles of incubation at 60°C. The resulting clone was mutagenized by direct treatment with N-methyl-N'nitro-N-nitrosoguanidine (NTG) or incorporation of the base analogues 5-azacytidine (5-Aza C) or 5-fluorouracil (5-FU) into replicating viral RNA.

Several of the resulting mutants were evaluated to determine complementation grouping, growth at 30°C, 37°C and 40°C, and virulence for 3 week old mice.

Complementation analysis of the mutant population indicates that at present five different complementation groups can be determined. Complementation ratio range from 0.09, indicating viral interference to 43.

Virus growth at different temperatures indicates that a spectrum of responses are seen. Mutant 200f is severely restructed while 100-351 is much less so. The growth curve for mutant 100-02 shows an intermediate pattern of growth inhibition at 40°C.

The results of virulence studies separate the mutants into two different groups. One group behaves in a manner similar to wild type while the second group appears to be avirulent.

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SUMMARY

Saint Louis encephalitis temperature-sensitive mutants derived by induction with 5-azacytidine and 5-fluorouracil have been examined to determine (1) complementation grouping, (2) growth characteristics at several temperatures and (3) virulence for mice.

The results indicate that a maximum of five tentative complementation groups may be established. One mutant has been placed in groups I and II and may represent a double mutant.

Examination of the ability of mutants to grow at 30°C, 40°C or 37°C indicates that some mutants are severely restricted at the nonpermissive temperature (40°C) while others are less so.

Virulence studies show a separation between avirulent mutants and those with a virulence quite similar to wild type. Mutants with a restricted growth in cell culture and a mutant with intermediate growth in cell culture were of greatly reduced virulence and behaved similarly when injected into mice.

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I. Statement of the problem:

The 1977 report from the Center for Disease Control indicated that of the 2,599 cases of encephalitis associated with viral illness 2,113 or 81% were the result of togaviral infections. SLE virus was confirmed as the cause of 86% with 142 resulting in death. These statistics emphasize the fact that SLE has been considered to be the prime cause of viral encephalitis in the United States.

Presently, no vaccine is available to protect against St. Louis encephalitis (SLE) or many other alpha or flavivirus infections.

The systematic production and evaluation of strains with potential to protect a given population is essential.

II. Background

Most live virus vaccines are composed of an attenuated population of viruses. The process of attenuation usually involved continuous passage in an animal or cell culture. This process results in the uncontrolled production of viruses with a reduced virulence. More recently, an examination of viruses with an altered ability to replicate at increased temperatures has resulted in the observations that they, in many cases, are less pathogenic (Ghendon, et al., 1973, Wagner, 1974, Brown et al., 1975).

A careful examination of ts mutants has suggested that these may, in fact, be good candidates for attenuated, live virus vaccines. The results of Ghendon (1973) indicate that a large number of polio virus ts mutants producing a pathologic change in infected monkeys were assayed for virus production. Results of assaying the isolated virus at permissive and nonpermissive temperatures indicates that a great deal of reversion is seen, with the isolated virus reflecting the temperature profile of the parental "wild type" virus.

However, the results presented by Brown, et al., (1975) indicate that when the ts mutants are carefully selected the virus seen in tissues are not the result of reversion but are the product of limited virus replication at body temperature.

The results of Wagner (1974), Brown, et al., (1975) and Harrison, et al., (1977) indicate that ts mutants used as live virus vaccines will confer protective immunity. One author (Wagner, 1974) suggests that the immunity observed in animals infected with vesicular stomatitis virus RNA^-, ts mutants is the result of residual protein synthesis (Wunner and Pringle, 1972). Brown, et al., (1975) tested a number of Eastern encephalitis virus ts mutants and reported tne ts mutants, mainly RNA mutants, would provide protection against challenge by the wild type virus. More recently investigators have begun to examine the ability of induced temperature-sensitive mutants to infect and replicate within animals. Barrett and Atkins (1979) have examined the virulence of mutants originally induced and isolated by Atkins, et al., (1974). Their results indicate that some mutants are avirulent while some maintain a high level of virulence. Their results also suggest that the phenotype of the mutant provided a gage as to the time required for the virus to kill its host. Mutants defective in RNA synthesis demonstrated an increase in the time necessary to kill mice. However, in most cases, the development of a lethal infection is determined by the ability of the mutant to revert to wild type.

Similar conclusions were drawn by Tan and Lubiniecki (1976) and Barrett and Atkins (1979). Their results indicate that ts mutants often exhibit reduced virulence for mice. However, it was also shown that temperature-sensitivity and mouse virulence could be separated (Tarr and Lubiniecki, 1976). These authors suggest that this might relate to the critical shut-off

temperature for a particular mutant. Some mutants may have shut-off temperatures below the 40°C level and thus display reduced virulence *in vivo* and *in vitro*. Others may not shut-off at body temperature but be obviously restricted *in vitro*. These experiments suggest that *ts* mutants may be a valuable source of viruses for vaccines.

III. Approach to the problem:

At the present time, there is no vaccine in general use to protect against an infection by SLE nor many of the other togaviral diseases. An effort needs to be made to identify the characteristics of effective vaccine strains of SLE. This information could result in not only selecting a vaccine strain of SLE but in selecting candidate vaccine strains of other togaviruses.

The nature of the viral vaccine used is of great importance. Information from studies with Poliovirus has shown that the use of live attenuated virus vaccines result in longer lasting, more effective immunity to challenge by the wild type virulent, virus.

In addition, inactivated vaccines for the togavirus Dengue have been shown to be ineffective (Tarr and Lubiniecki, 1976). This proposal is an effort to systematically develop and select attenuated viral strains with the characteristics of good vaccines. These characteristics are (1) few or no symptoms upon infection (2) induction of long lasting protective immunity against wild type virus (3) and no reversion to the wild type. Temperature-sensitive mutants may provide effective protection against challenge by wild type strains.

IV. Results

A. Complementation

Theoretically, conditioned lethal temperature-sensitive mutants may be

derived which represent all possible gene products. The relatedness of temperature-sensitive mutants may be determined by their ability to share functions and produce virions under nonpermissive conditions. Virions complementing one another are necessarily defective in differing gene products. The examination of all possible paired combinations of mutants should provide information about the number of gene functions expressed and group virions on the basis of similar defects.

During the past year, many mutants have been examined in an effort to determine genetic relatedness. The results of complementation analysis to date are presented in Table 1. These results indicate a range in the complementation ratios from 0.09 to 43. Mixed infection with several mutants resulted in ratios much less than one, suggesting that viral interference has taken place. In contrast combinations of mutants, such as 100-351 and 100-352, produce larger numbers of progeny during a mixed infection than when each mutant singly infects cells. Complementation is generally considered to have occured when a complementation index of two or greater is seen.

The results from Table 1 indicate that several of the mutants belong to different groups. The results allow the establishment of a maximum number of complementation groups. The tentative groups, I through V, and the numbers of each group are presented in Table 2. This assignment to tentative complementation groups represents the maximum number of complementation groups possible. Some rearrangement may occur as the ultimate number of paired combinations is approached

One mutant, 100-35, appears in complementation groups I and II. The reason for this is unclear. Mutant 100-35 may be a double mutant, incapable of complementing with group I or group II mutants. However, it may be that the mixed infection between 100-35 and 100-0_2 was inefficient resulting in an incorrectly low complementation ratio.

TABLE 1

Complementation Frequencies

STRAINS

STRAINS	25e	100-0	$D_2D_1$1000g	$D_2$1000i	100-35	100-0_2	100-0_1	100-351	100-352	200f	100-2	100-02
25e	-	1.00	.608	1.042			1.27					
100-0		-								.37		
$D_2D_1$100g			-									
$D_2$1000i				-			.610					
100-35					-	1.13				.35		
100-0_2						-	3.6					
100-0_1							-					
100-351								-	43		2.06	
100-352									-			
200f										-	2.24	4.8
100-2											-	.09
100-02												-

TABLE 2

Complementation Groups

Group	Mutants
I	25e, 100-O_1, $D_2$1000i, $D_2D_1$1000q 100-0, 200f, 100-35
II	100-O_2, 100-35
III	100-351
IV	100-352
V	100-2, 100-02

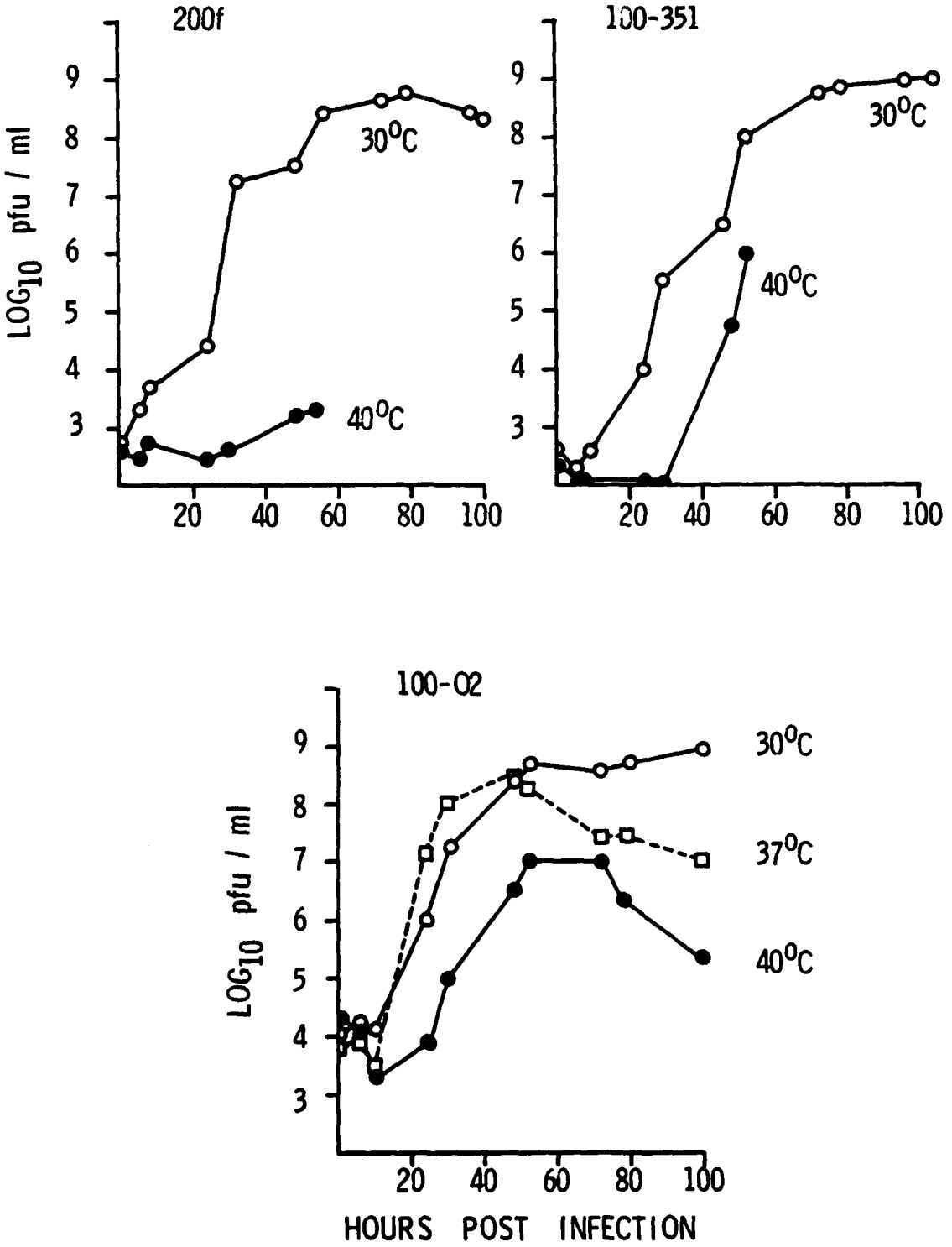
The results with combinations such as 100-352 and 100-351 or 100-02 and 200f indicate that complementation can be observed between temperature-sensitive mutants of St. Louis encephalitis.

B. Growth of mutants at selected temperatures

Data from other investigators have linked temperature sensitivity to reduced virulence while allowing the induction of a protective immune response (Eckels, et al., 1976; Harrison, et al., 1977; Tarr and Lubiniecki, 1976; Barrett and Atkins, 1979). However, the simple measurement of temperature sensitivity is not an adequate predictor of attenuation and antigenicity. The ability of a temperature-sensitive mutant to grow at internal body temperature may have a significant effect on the ability of the virus to induce a protective immune response or cause significant damage to the host. Not all temperature-sensitive mutants possess the same "shut off" temperature, or temperatures at which replication is significantly restricted.

As part of an overall effort to understand viral pathogenesis and how temperature-sensitive lesions may effect it, the ability of several mutants to grow at 30, 37 and 40°C was examined. The results are presented in Figure 1. These data provide some information about the ability of the virus to grow under different environmental pressures. Mutants such as 200f are greatly restricted at 40°C up through 54 hours post infection. A different pattern of replication is seen when 100-351 is grown at 30°C and 40°C. Mutant 100-351 shows a pattern of restricted growth through 30 hours post infection. However, subsequent replication parallels that seen at 30°C. A third pattern of response to temperature is seen with mutant 100-02. The results show an intermediate amount of replication at 40°C with increasing amounts of virus produced at 37°C and 30°C. The significance of these data is not clear at the present time.

Figure 1. Growth curves of three temperature-sensitive mutants. Cells were inoculated with virus and adsorbed at 4°C for 1-2 hours. After adsorption, prewarmed media was added and cells were incubated at the appropriate temperatures. Samples taken at the times indicated were assayed by plaque formation on PS-2 cell monolayers.


200f
100-351
100-02
LOG10 pfu / ml
30°C
37°C
40°C
HOURS POST INFECTION

These data may provide valuable information when combined with animal virulence studies of each mutant. While simple selection of temperature-sensitive mutants may not assure that they will be adequate vaccine strains, examination of replication at selected temperatures may improve our ability to predict their success.

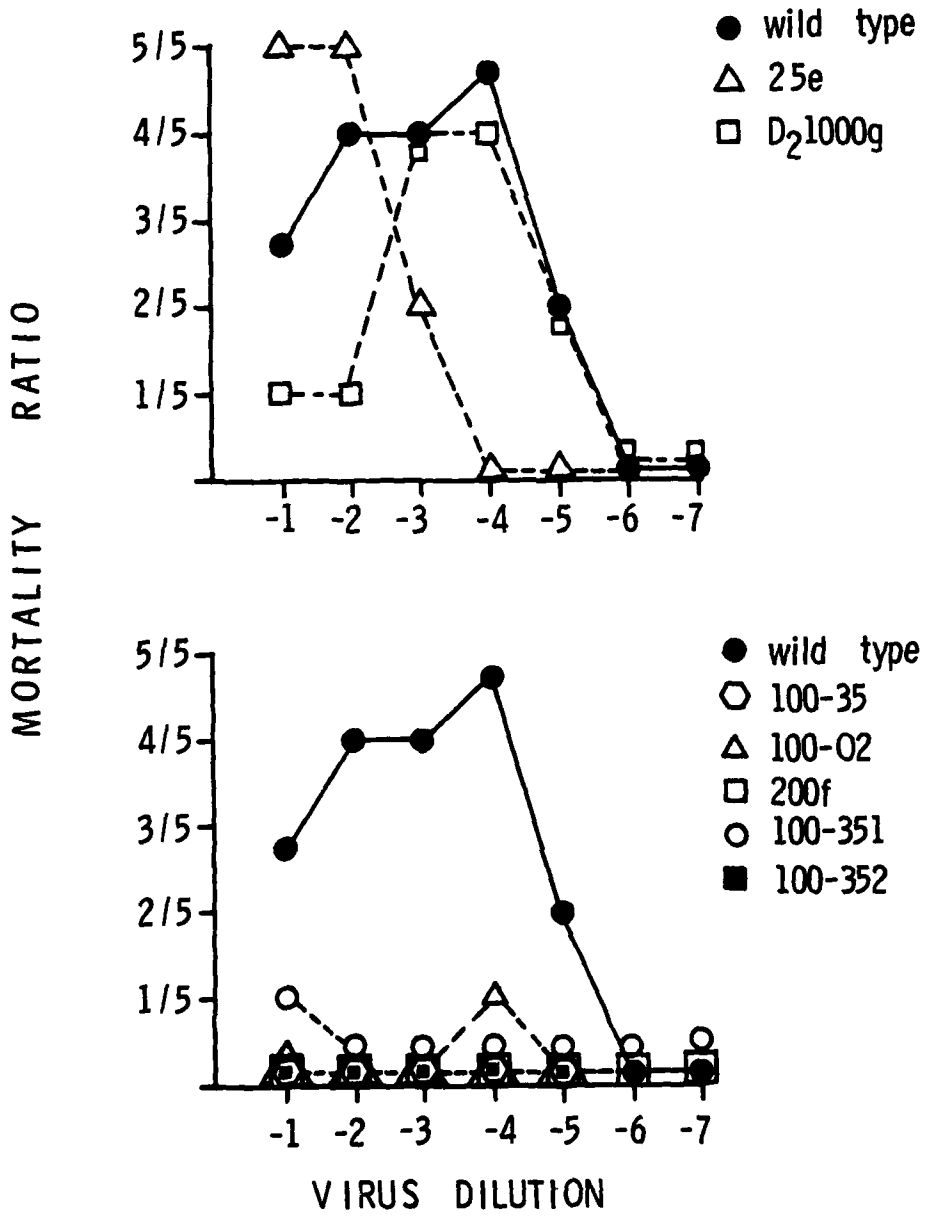
C. Virulence for mice

Saint Louis encephalitis virus has been shown by others to be virulent for three week old random bred Swiss Webster mice (Vector-borne disease, 1977). This provides an excellent opportunity to compare the lethality of wild type and the attenuation of selected mutants. The results of these experiments are presented in Figure 2. The experiments were designed so that five mice were injected intraperitoneally with 0.2 ml of the appropriate virus dilution. Noninfected, sham inoculated controls, as well as mice inoculated with dilutions of the wild type stock virus, were included with each new set of experiments. Each group was observed daily for fourteen days and the number of mice dead on each day was recorded. This allowed the calculation of an average day to death, mouse $IPLD_{50}$ and pfu/LD_{50} for each mutant. As the results indicate, the mutants fall into two groups. This first group, containing the majority of mutants, was of greatly reduced virulence. The second group, containing two mutants, was as virulent as the wild type virus. A summary which includes the pfu/LD_{50} for each mutant is presented in Table 3.

The results indicate that mutants with relatively low EOP's demonstrate a low virulence for mice, while those with high EOP's are similar to wild type virus in their virulence.

Mouse lethality is a crude monitor of virus replication within the host. It is imperative that the amount and location of virus replication, and the

Figure 2. Mortality ratio for temperature-sensitive mutants of Saint Louis encephalitis virus. Each mutant was diluted to the same titer and 0.2 ml of each subsequent ten fold dilution was injected. Each dilution was injected into five mice. The mice were observed each day for 14 days. The number of dead mice are presented in these graphs. In the upper and lower panel, wild type virus is represented by the filled circles.


MORTALITY RATIO
5/5
4/5
3/5
2/5
1/5
-1 -2 -3 -4 -5 -6 -7
wild type
25e
D2 1000g
wild type
100-35
100-02
200f
100-351
100-352
VIRUS DILUTION

characteristic of the virus produced, be determined even when survival of the host is obvious.

The data presented in Table 3 are the results of many mouse virulence experiments. However, they are only a small portion of the number planned for this year. The virulence experiments were abruptly halted when outside temperatures reached 100°F. Mice shipped into Columbia were arriving in poor health or dead. As a result, experiments with mice have stopped. They will be restarted when the temperature drops.

V. Conclusions and recommendations

A. Conclusions

Three characteristics of several temperature-sensitive mutants have been examined to date: (1) Complementation analysis, (2) growth at selected temperatures and (3) relative mouse virulence of selected mutants.

1. Complementation: The results indicate that complementation does occur and may be used as a mechanism for grouping mutants. Complementation analysis has allowed the establishment of five groups. The largest number of mutants belong to group I. The function of each group is still unknown.

2. Growth at several temperatures: The data accumulated to date indicates that mutants vary in the degree to which they replicate at the nonpermissive temperature. This information may prove to be helpful in the selection of immunogenic yet nonvirulent strains. However, in order to make this judgement, one must correlate data from growth curve with information on virulence and antibody production resulting from inoculation. Virus which does not grow well at 37°C or higher temperatures may well be avirulent. It may also be nonimmunogenic due to a total lack of replication.

3. Virulence: Mutants 200f, 100-02, 100-35, 100-351 and 100-352 have shown a lack of mouse virulence when evaluated by i.p. injection of 3 week

Table 3

Summary of data on temperature-sensitive mutants

MUTANT STRAIN	EOP[a]	Complementation Group[b]	RNA Phenotype	LD_{50}[c] (pfu)	Mean Day of Death (± s.d.)
Wild type	1.02	-	+	3,206	10.24 (± .61)
100-35	2.0×10^{-4}[f]	I, II	+	$>10^6$	All survived
100-351	2.0×10^{-4}	III	ND[d]	$>10^6$	All survived
100-352	3.0×10^{-4}	IV	ND	$>10^6$	11[e]
200f	5.0×10^{-7}[f]	I	ND	$>10^6$	All survived
$100\text{-}0_1$	5.7×10^{-6}	II	-	ND	ND
$100\text{-}0_2$	$<1.4 \times 10^{-8}$	II	-	ND	ND
100-0	$<4.2 \times 10^{-8}$	I	ND	ND	ND
25e	9.0×10^{-1}[f]	I	ND	176	10.0 (± 1.48)
D_2 1000i	4.1×10^{-3}	I	ND	ND	ND
D_2D_1 1000g	1.2×10^{-2}	I	ND	5,400	11.4 (± 1.12)
D_2 1000c[g]		ND	ND	80	9.7 (± .99)
100-02	2.0×10^{-7}	V	ND	$>10^6$	All survived
100-2	1.4×10^{-5}[f]	V	ND	ND	ND

[a] EOP = Efficiency of plating $\frac{\text{pfu/ml @ } 40^{\circ}C}{\text{pfu/ml @ } 30^{\circ}C}$

[b] Tentative complementation group

[c] Determined in 3 week old Swiss Webster mice. Each mouse inoculated with 0.2 ml i.p. and observed for 14 days

[d] Not done

[e] Only one mouse died

[f] Regrowth of original stock

[g] Additional mutant

old mice. In the case of 200f and 100-02, this correlates with a reduced ability to grow at 40°C in cell culture. Whether or not these mutants are inducing an immune response is unknown. Serum samples have been obtained from mice inoculated with these mutants. Examination of the neutralization potential of these sera will provide information about the protective potential of each mutant.

4. General: A comparison of the growth rates of mutants 200f and 100-351 indicate two different reactions to incubation at 40°C. Mutant 200f is severely restricted at 40°C while 100-351 is less restricted. However, both of these mutants were determined to be avirulent when assayed in 3 week old mice. The reason for this is not presently clear. Replication in vito and in vivo may well be quite different. Or the in vitro results may be an accurate reflection of virus growth in the host, but even the levels of replication seen with 100-351 may be below the threshold need to establish a pathogenic state. Examination of virus replication within the inoculated host may help to answer this important question.

It is not known whether extensive replication within the host is essential for an expressed or protective antibody response. Determination of virus replication after one inoculation and corelation with antibody levels may help to determine the optimum virus inoculum.

B. Recommendations for continued research

These results suggest that future research should investigate the replication of mutants in the host. The amount of virus produced and the characteristics of this virus should be examined by isolating various organs, extracting virus and assaying at permissive and nonpermissive temperatures.

In addition, the immune response both homologous and heterologous, to injection with temperature-sensitive mutants should be examined.

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